

Selective FGFR inhibitor BGJ398 inhibits phosphorylation of AKT and STAT3 and induces cytotoxicity in sphere-cultured ovarian cancer cells

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Received December 3, 2016; Accepted March 3, 2017

DOI: 10.3892/ijo.2017.3913

Abstract. Epithelial ovarian cancer is the most aggressive and lethal among the gynecological malignancies, which is often found disseminated to peritoneal cavity at the time of diagnosis. There is accumulating evidence on the existence of genetic alteration and amplification of fibroblast growth factor receptor (FGFR) in various cancers. Also the aberrated FGFR/FGF signaling has been implicated in cancer development and tumor microenvironment. However, the antitumor activity of BGJ398, a selective inhibitor of FGFR 1/2/3 against ovarian cancer still remains unknown. The aim of the present study is to evaluate the antitumoral activity of BGJ398 on ovarian cancer cell line SKOV3ip1 using 3-dimensional (3D) sphere culture system which has been accepted as a better mimic *in vivo* microenvironment than conventional 2-dimensional (2D) monolayer culture system. We examined the differential expression features of key signaling molecules which have a role in cell survival and proliferation between sphere-cultured SKOV3ip1 cells and monolayer-cultured SKOV3ip1 cells. The phosphorylation of AKT and signal transducer and activator of transcription 3 (STAT3) known as survival signaling molecules were upregulated in sphere-cultured SKOV3ip1 cells compared to in monolayer-cultured SKOV3ip1 cells. Next, we evaluated the antitumor activity

of BGJ398 in monolayer-cultured SKOV3ip1 cells or sphere-cultured SKOV3ip1 cells. Treatment of BGJ398 did not affect the SKOV3ip1 cell viability in monolayer culture system, but, the cell viability of sphere-cultured SKOV3ip1 cells was markedly reduced by BGJ398. The phosphorylation of AKT and STAT3 was downregulated by BGJ398 in sphere-cultured SKOV3ip1 cells, but not in monolayer cultured-SKOV3ip1 cells. Moreover, combination treatment with BGJ398 and paclitaxel in sphere-cultured SKOV3ip1 showed synergistic inhibitory effect on cell viability. Collectively, our report reveals the BGJ398 is a potent antitumor agent against ovarian cancer and FGFR is a promising therapeutic target to anticancer therapy considering ovarian cancer metastatic microenvironment.

Introduction

Epithelial ovarian carcinomas (EOCs) account for 90% of total cases of ovarian cancer. Unfortunately, almost 70% of women with ovarian cancer are not diagnosed until the disease is advanced in stage leading to the highest mortality of any of the gynecologic cancers (1). Paclitaxel has been used as a first-line chemotherapeutic agent against ovarian cancer (2). However, the development of chemoresistance causes the primary failure in the treatment of ovarian cancer (3). Although improvement in survival rate has been observed, the majority of patients experience recurrent disease due to resistance to chemotherapy (4). The identification of suitable biomarkers for chemosensitivity diagnosis to paclitaxel and the development of new therapeutic agent for overcoming chemoresistance may be important research fields to improve the therapeutic outcome of patient with ovarian cancer.

The fibroblast growth factor receptors (FGFRs) consist of an extracellular ligand domain with three immunoglobulin-like domains (I-III), a transmembrane domain, and an

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Key words: BGJ398, ovarian cancer, sphere culture, AKT, STAT3, FGFR

intracellular tyrosine kinase domain that transmit the signal to the intracellular binding proteins (5). There are four FGFRs (FGFR1/2/3/4) as membrane-bound receptors with seven isoforms due to the alternative splicing in Ig-III-like domain with different ligand-binding affinity (5). While each FGFR can be activated by several FGFs, the FGF/FGFR signaling is further controlled with different tissue distribution (5,6). For example, high level expression of FGFR1 was observed in the skin, cornea, lung, heart, placenta, kidney and uterus. In contrast, high expression of FGFR2 was detected in prostate and stomach. The expressions of FGFR3 and FGFR4 were more restricted than FGFR1 and FGFR2. FGFR3 expression is found in appendix, colon, liver, sublingual gland, placenta, and cervix with restricted and lower patterns. FGFR4 expression is observed in the liver, sublingual gland duct, kidney and ureter (6). Differential tissue distribution of FGFR expressions suggests that FGFR families play important functional roles in normal tissue homeostasis (7). Recent studies have indicated that FGFR signaling is also implicated in tumor development (8-10). Cumulative evidence revealed that FGFR aberrations are common in a wide variety of cancers, with the majority being gene amplification or activating mutations (11). Helsten *et al* (12) reported that FGFR aberrations were found in 7.1% of cancers using next-generation sequencing. Moreover, ovarian cancer is the fifth most commonly altered (~9%) in FGFR activity among the cancer malignancies implicating that FGFR inhibition could be an important therapeutic approach against ovarian carcinoma (12).

AKT/PKB is an intracellular serine/threonine kinase. It has been extensively studied that AKT regulates a variety of cellular processes by mediating extracellular and intracellular signals (13-16). Activation of AKT mediates its pleckstrin homology (PH) domain binding to products of phosphatidylinositol 3-kinase (PI3K). This activation is feedback regulated by a dual phosphatase PTEN (17). Activation of AKT through platelet-derived growth factor (PDGF), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) has been identified (18-21). These events regulate cell growth, survival, differentiation, angiogenesis, migration and metabolism (22). Moreover, studies have shown that AKT signaling is frequently impaired in many malignancies (23) and the overexpression of AKT induces chemoresistance (24). To date, many studies have demonstrated that AKT signaling is the major target for anticancer drug development and overcoming chemoresistance.

Three-dimensional sphere culture model, a strategy with cell anchorage-independent growth potential, have been reported to establish reliable methodologies and techniques for the high-throughput drug development against various cancers (25-27). Although the sphere culture model is a better recapitulating system of primary tumors than conventional monolayer culture model, the sphere culture model approaches have not yet been used widely in cancer research and drug development fields because of higher costs and technical problems than conventional monolayer culture model (28). The wide application of sphere culture model in cancer cell research and the accumulation of available data through the sphere culture model may facilitate the understanding of tumor growth and metastasis in specific tumor microenvironment (29,30). Especially, epithelial ovarian cancer frequently

spreads by direct metastasis from the primary site (31). Unlike primary tumor in ovary, premetastatic ovarian cancer cells undergo epithelial-to-mesenchymal (EMT) transition, which loosens the intercellular attachment between cancer cell-to-cell and cancer cell-to-extracellular matrix through the remodeling of cadherins (31). Detached ovarian cancer cells from the primary site disseminate within the abdominal cavity and often associated with ascitic fluid, particularly in high-grade serous carcinoma. In addition, the disseminated ovarian cancer is faced with a harsh growth condition with the specific tumor microenvironment involving hypoxia and nutrient-deprived conditions (31). Nonetheless, to date the conventional monolayer ovarian cancer culture model has been used for ovarian cancer research preferentially. Here, we report the molecular characterization of ovarian carcinoma SKOV3ip1 cell line in conventional monolayer culture model and sphere cultured model. We evaluated the antitumor activity of the selective FGFR inhibitor BGJ398 against SKOV3ip1 in sphere culture model. These data suggest that BGJ398 is a potent anticancer drug candidate against epithelial ovarian carcinoma. The present data also showed that the inhibition of FGFR/AKT signaling pathway represents a therapeutic target for overcoming metastatic ovarian carcinoma.

Materials and methods

Antibodies and reagents. Antibodies against STAT3 (H-190), phosphorylated STAT3 at Tyr 705 (B-7) and Actin (C-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal antibodies against AKT, phosphorylated AKT at Ser473 (D9E), phosphorylated FGFR1 at Tyr 653/654, p42/44 MAPK, phosphorylated p42/44 MAPK, p38 MAPK and phosphorylated p38 MAPK at Tyr 180/182 antibodies were from Cell Signaling Technology (Danvers, MA, USA). Goat anti-mouse and rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). BGJ398 (against FGFR1/2/3), TAE684 (against ALK) and imatinib (against Abl) were purchased from Selleck Chemicals LLC (Houston, TX, USA). AG490 (against JAK) was from Tocris Bioscience (Bristol, UK). All reagents were solubilized with dimethyl sulfoxide (DMSO) at the following concentration, paclitaxel at 100 μ M, BGJ398 at 5 mM, TAE684 at 3 and imatinib at 5 mM.

Cell lines and sphere culture. SKOV3ip1 ovarian carcinoma cell line (32) was a kind gift from Professor A.K. Sood (University of Texas MD Anderson Cancer Center, Houston, TX, USA). Cells were maintained as a 2D monolayer in RPMI-1640 medium (Corning Incorporated, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 10 U/ml penicillin/streptomycin (Gibco) at 37°C in a 5% CO₂ humidified incubator. To culture as 3D sphere system, SKOV3ip1 cells (1x10⁶) were seeded in ultra-low attachment 6-well plate (Corning Incorporated) and were cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco) and 10 U/ml penicillin/streptomycin (Gibco) at 5% CO₂ incubator.

Immunoblotting. The expression of protein and their phosphorylation status in SKOV3ip1 cells was detected by

immunoblotting. Monolayer-cultured or sphere-cultured SKOV3ip1 cells were harvested and washed once with ice-cold 1X phosphate-buffered saline (PBS), and then lysed by 100 μ l ice-cold RIPA buffer (20 mM Tris-Cl, pH 8.0, 125 mM NaCl, 100 mM phenylmethylsulfonyl fluoride, 1 mM ethylene diamine tetraacetic acid, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1X Complete Protease Inhibitor Cocktail) (Roche, Mannheim, Germany) on ice. Protein concentrations were determined using Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amount of proteins (30 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes, followed by immunoblotting with the specific antibodies and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) using Fusion Solo chemiluminescence analyzer (Vilber Lourmat, Marne la Vallee, France).

Cell viability assay. The crystal violet staining assay was examined to determine the cell viability in monolayer culture and sphere culture model. To examine the crystal violet staining assay in monolayer culture model, SKOV3ip1 cells (5×10^4) were seeded in conventional 24-well plate and incubated overnight. The drugs were then treated with the following concentration to each well; vehicle control (DMSO); paclitaxel at 3.125, 6.25, 12.5, 25, 50 and 100 nM, BGJ398 at 0.3125, 0.625, 1.25, 2.5 and 5 μ M, TAE684 at 0.1875, 0.375, 0.75, 1.5 and 3 μ M, and imatinib at 0.3125, 0.625, 1.25, 2.5 and 5 μ M. Cells were further incubated for 72 h. Three hundred microliter 0.2% crystal violet solution was added to each well and further incubated for 20 min with gently shaking. Stained cells were washed with distilled water until a clear background was visible. For colorimetric analysis, crystal violet dye in each well was extracted using 1% SDS/PBS solution and the absorbance was determined using EMax PLUS microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm wavelength. For determination of cell viability in sphere-cultured cells, SKOV3ip1 cells (5×10^4) were seeded in ultra-low attachment 24-well plate and incubated overnight. Then drugs were added and the cells were further incubated for 72 h. Cells in each well were harvested and transferred to conventional 24-well plate and further incubated for 12 h. Attached viable cells were stained by 0.2% crystal violet solution for 20 min with gently shaking. Stained cells were washed with distilled water. For colorimetric analysis, crystal violet dye was extracted using 1% SDS/PBS then the absorbance was determined at 570 nm using EMax PLUS microplate reader (Molecular Devices).

MTS assay. The cell viability was analyzed by MTS assay in monolayer culture and sphere culture model. For the MTS assay, EZ-Cytox (Daeilab Service, Seoul, Korea) was used following the manufacturer's instructions. SKOV3ip1 cells were seeded into conventional 96-well plate or ultra-low attachment 96-well plate at a density of 1×10^4 /well and incubated overnight. The drugs were then treated with the following concentration to each well; vehicle control (DMSO); paclitaxel at 3.125, 6.25, 12.5, 25, 50 and 100 nM, BGJ398 at 0.3125, 0.625, 1.25, 2.5 and 5 μ M, TAE684 at 0.1875, 0.375, 0.75, 1.5

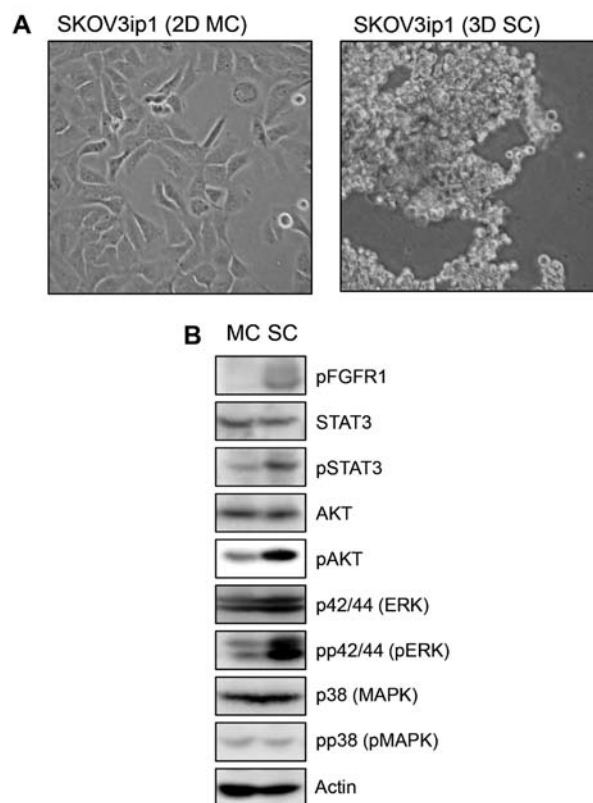


Figure 1. Differential expression pattern of signal molecules in cell survival between monolayer-cultured and sphere-cultured SKOV3ip1 ovarian cancer cells. (A) Monolayer-cultured and sphere-cultured SKOV3ip1 cells were visualized using microscopy. (B) SKOV3ip1 cells (1×10^6) were seeded in conventional plate or ultra-low attached plate and further incubated for 72 h. Cell lysates from monolayer-cultured (2D MC) SKOV3ip1 cells and sphere-cultured (3D SC) SKOV3ip1 cells were subjected to immunoblotting to analyze the expression level of AKT, STAT3, pp42/44 ERK and p38 MAPK and its activation status. The phosphorylated FGFR1 at Tyr 653/654 residues was also examined.

and 3 μ M, and imatinib at 0.3125, 0.625, 1.25, 2.5 and 5 μ M. Cells were further incubated for 72 h. Sphere-cultured cells in ultra-low attachment 96-well plate were harvested and transferred to conventional 96-well plate and further incubated for 12 h. Wells with culture media were used as negative control. The absorbance was determined at 450 nm using EMax PLUS microplate reader (Molecular Devices).

Statistical analysis. All statistical analyses of data were performed from three independent experiments. All experiments were repeated three times. Significant differences by concentration were calculated using one-way ANOVA test and $P \leq 0.05$ analyzed by concentration were considered significant. All graphs showed the mean with the standard deviation. Cell viability data were analyzed with GraphPad Prism 6 statistical software and presented as the mean value of viable cells \pm standard deviation (SD).

Results

Differential expressions of survival signaling molecules in SKOV3ip1 ovarian cancer cell cultured in conventional 2D monolayer culture model and 3D sphere culture model. We examined the morphological analysis of SKOV3ip1 ovarian

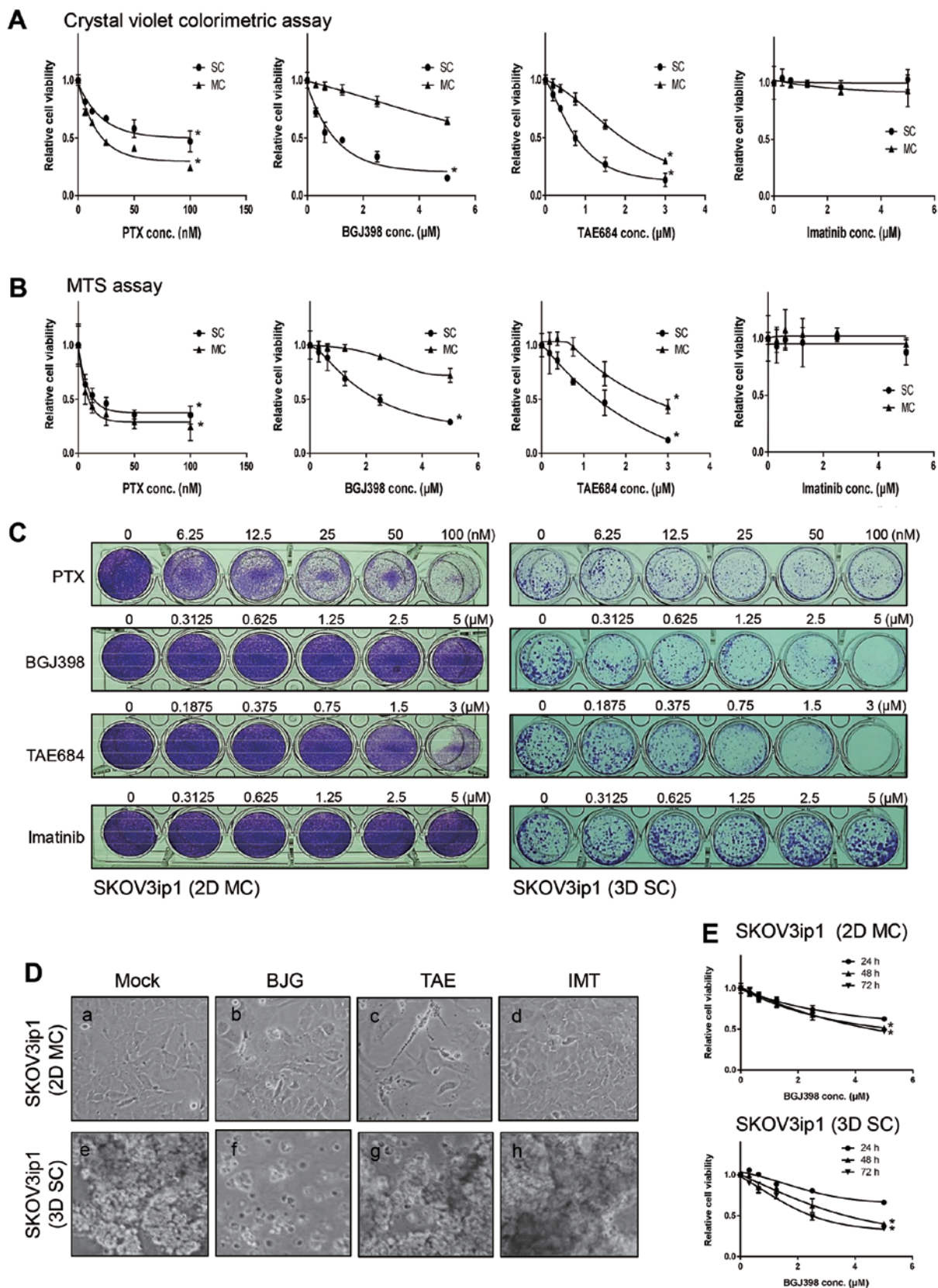


Figure 2. Selective FGFR inhibitor BGJ398 reduces the cell viability in sphere-cultured SKOV3ip1. Cells (5×10^4) were seeded in conventional 24-well plate and incubated overnight. Then drugs were added with the following concentration to each well; vehicle control DMSO (mock); paclitaxel at 3.125, 6.25, 12.5, 25, 50 and 100 nM, BGJ398 at 0.3125, 0.625, 1.25, 2.5 and 5 μ M, TAE684 at 0.1875, 0.375, 0.75, 1.5 and 3 μ M, and imatinib at 0.3125, 0.625, 1.25, 2.5 and 5 μ M. Cells were further cultured for 72 h and the cell viability was determined by crystal violet colorimetric analysis (A) and MTS assay (B). (C) Viable cells at 72 h after treatment paclitaxel or inhibitors as indicated concentration were visualized by crystal violet staining. (D) Monolayer (2D MC) or spheroid formation (3D SC) of SKOV3ip1 at 72 h after treatment of vehicle solution DMSO (Mock), 5 μ M BGJ398 (BGJ), 3 μ M TAE648 (TAE) and 5 μ M imatinib (IMT). (E) SKOV3ip1 cells (5×10^4) were seeded in conventional 24-well plate and incubated overnight. Then DMSO (mock) or BGJ398 (0.3125, 0.625, 1.25, 2.5 and 5 μ M) were added to each well. The cell viability was determined by crystal violet colorimetric analysis at 24, 48 and 72 h. All experiments were repeated three times. Significant differences by concentration were calculated using one-way ANOVA test and $P \leq 0.05$ analyzed by concentration were considered significant.

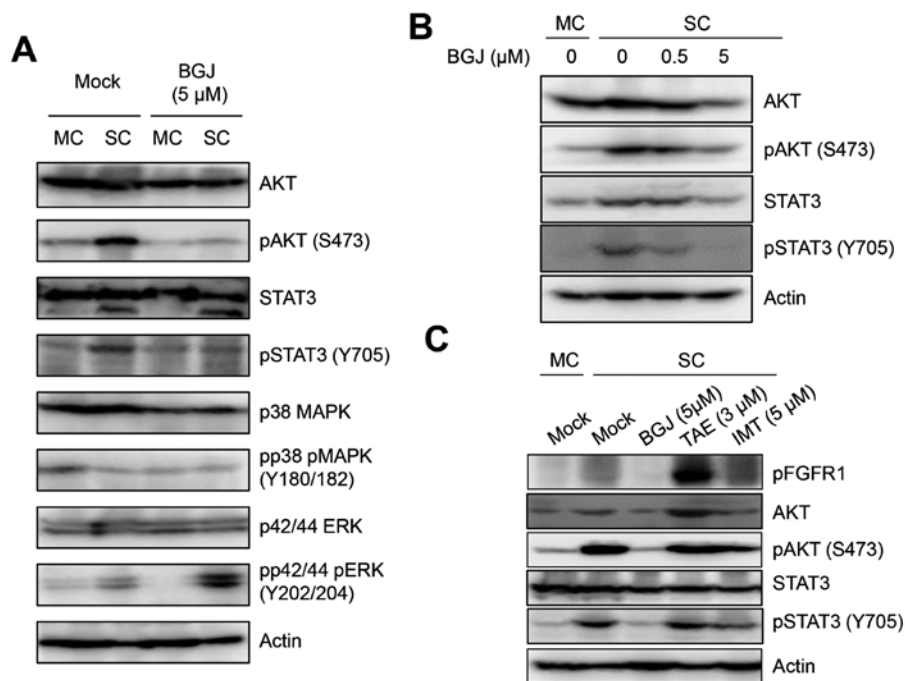


Figure 3. BGJ398 suppresses the phosphorylation of AKT and STAT3 in sphere-cultured SKOV3ip1. (A) Monolayer-cultured (MC) and sphere-cultured (SC) SKOV3ip1 cells were treated with DMSO (Mock) and 5 μ M BGJ398, respectively. At 72 h after treatment, cells were harvested and were analyzed the activation status of AKT, STAT3, p38 MAPK and p42/44 ERK by immunoblotting. (B) SKOV3ip1 cells were treated with DMSO (Mock) and 0.5 and 5 μ M BGJ398, respectively. At 72 h after treatment, immunoblotting was performed to analyze the cellular expression and activation of AKT and STAT3 with indicated antibodies. (C) Cell lysates at 72 h after treatment of DMSO (Mock), 5 μ M BGJ398 (BGJ), 3 μ M TAE648 (TAE), and 5 μ M imatinib (IMT) in sphere-cultured SKOV3ip1 were subjected to immunoblotting and the cellular expression of AKT, STAT3 and its activation status were analyzed with indicated antibodies. The level of phosphorylated FGFR1 was shown as a positive control of BGJ treatment. Actin was used as a loading control.

cancer cells in conventional 2D monolayer culture and 3D sphere culture model. At 72 h after sphere cultivation, SKOV3ip1 cells formed loose sheet-like aggregates and did not accumulate as compact spheroids (Fig. 1A). We examined the expression of cellular signaling molecules which have a role in cell proliferation including AKT, STAT3, p42/44 ERK and p38 MAPK and their activation status in monolayer-cultured SKOV3ip1 cells and sphere-cultured SKOV3ip1 cells. Immunoblotting showed that the expression level of molecules was not different in monolayer-cultured SKOV3ip1 cells and sphere-cultured SKOV3ip1 cells. Notably, p42/44 ERK, AKT and STAT3 were activated in sphere-cultured SKOV3ip1 cells than in 2D monolayer-cultured SKOV3ip1 cells (Fig. 1B). Additionally, phosphorylated FGFR1 was upregulated in sphere-cultured SKOV3ip1 cells (Fig. 1B). These results showed that the sphere culture condition upregulated the prosurvival signaling pathway in SKOV3ip1 cells.

Selective pan-FGFR inhibitor BGJ398 inhibits cell viability of SKOV3ip1 cells in sphere culture model. FGFR inhibitor has been reported as a novel agent with potential anti-angiogenic and anticancer effect (33). It has been reported that FGFR signaling could regulate PI3K/AKT and STAT3 pathway in mammalian cells (34). Thus, we examined the effect of a highly selective pan-FGFR inhibitor BGJ398 on SKOV3ip1 cell viability. Tyrosine kinase inhibitor TAE648 (against ALK) and imatinib also known as Gleevec (against ABL), and standard anti-ovarian cancer chemotherapeutic agent paclitaxel were used and compared in antitumor activities of SKOV3ip1 cells. Monolayer-cultured SKOV3ip1 cells were treated with

various concentrations [vehicle control (DMSO)]; paclitaxel at 3.125, 6.25, 12.5, 25, 50 and 100 nM, BGJ398 at 0.3125, 0.625, 1.25, 2.5 and 5 μ M, TAE648 at 0.1875, 0.375, 0.75, 1.5 and 3 μ M, and imatinib at 0.3125, 0.625, 1.25, 2.5 and 5 μ M) of tyrosine kinase inhibitors and paclitaxel for 72 h, and the cell viability was determined using crystal violet colorimetric analysis (Fig. 2A) and MTS assay (Fig. 2B). Visualization of crystal violet stained cells confirmed the result of colorimetric analysis (Fig. 2C). As shown in Fig. 2A and B, monolayer-cultured SKOV3ip1 cell viability was effectively decreased by paclitaxel and TAE648 in a dose-dependent manner ($P > 0.05$). But the cell viability of monolayer-cultured SKOV3ip1 was not affected by BGJ398 and imatinib (Fig. 2A). Next, the cell viability of sphere-cultured SKOV3ip1 by treatment of paclitaxel or tyrosine kinase inhibitors was compared. Cell viability analysis showed that sphere-cultured SKOV3ip1 cells were clearly more resistant to paclitaxel than SKOV3ip1 in monolayer (Fig. 2A). TAE648-resistance was not observed in sphere-cultured SKOV3ip1 cells (Fig. 2A). Also imatinib did not influence the cell viability in sphere-cultured SKOV3ip1 cells (Fig. 2A). Notably, BGJ398 differentially affected the cell viability in monolayer-cultured and sphere-cultured SKOV3ip1 (Fig. 2A and B). BGJ398 reduced the cell viability of sphere-cultured SKOV3ip1 cells in a dose-dependent manner ($P > 0.05$; Fig. 2A). MTS assay was used to confirm viability of cells treated with inhibitors. The inhibition of cell viability by BGJ398 was also observed in sphere cultured SKOV3ip1 cells ($P > 0.05$; Fig. 2B). BGJ398-treated sphere-cultured SKOV3ip1 cells did not maintain the healthy aggregate formation (Fig. 2D). Dissociation of spheroids were not observed

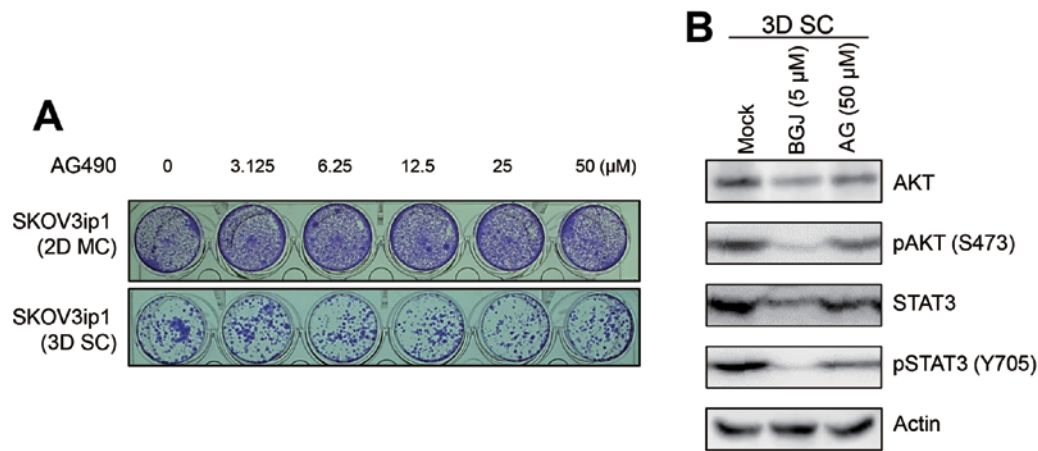


Figure 4. JAK inhibitor AG490 did not inhibit cell viability, nor downregulated the phosphorylation status of AKT and STAT3 in sphere-cultured SKOV3ip1 cells. (A) Cell viability of sphere-cultured SKOV3ip1 at 72 h after treatment with AG490 at the indicated concentration was analyzed by crystal violet staining. (B) Sphere-cultured SKOV3ip1 cells were treated with 5 μM BGJ398 or AG490 50 μM AG490 for 72 h. The expression and its phosphorylation of AKT and STAT3 were assessed by immunoblotting. Actin was used as a loading control.

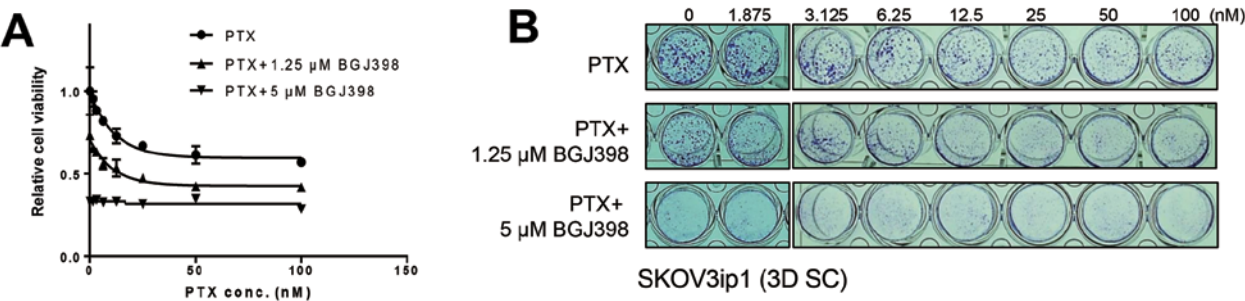


Figure 5. BGJ398 enhances the cell viability inhibition of sphere cultured SKOV3ip1 with the combination treatment of paclitaxel. (A) Cell viability of sphere-cultured SKOV3ip1 at 72 h after treatment with serial diluted paclitaxel (0, 1.875, 3.125, 6.25, 12.5, 25, 50 and 100 nM) together with dose-different combinations (0, 1.25 and 5 μM) of BGJ398 was determined by crystal violet colorimetric analysis. (B) Viable cells at 72 h after combination treatment of paclitaxel and BGJ398 at indicated concentration were visualized by crystal violet staining. All experiments were repeated three times. Significant differences by concentration were calculated using one-way ANOVA test and $P \leq 0.05$ analyzed by concentration were considered significant.

at TAE684-treated and imatinib-treated sphere-cultured SKOV3ip1 (Fig. 2D). Next, we examined the cell viability assay with BGJ398 at 24-72 h. The result showed that the cell viability was decreased in a dose-dependent manner both in monolayer cultured model and sphere cultured model at 24-72 h (Fig. 2E). Interestingly, decreased cell viability was not significant in monolayer-cultured SKOV3ip1 cell at 48- and 72-h treatments ($P > 0.05$) (Fig. 2E), but in sphere-cultured SKOV3ip1 cells, the cell viability was gradually decreased in a time-dependent manner (Fig. 2E). These data revealed that BGJ398 is a potent chemotherapeutic agent against SKOV3ip1 cells depending on sphere growth conditions.

BGJ398 inhibits the activated AKT and STAT3 in sphere-cultured SKOV3ip1 cells. Since major survival signaling molecules, AKT and STAT3 were activated in sphere-cultured SKOV3ip1 cells, we examined whether BGJ398 was able to affect the activation status of AKT and STAT3 in sphere-cultured SKOV3ip1 cells. Immunoblotting analysis indicated that BGJ398 suppressed the phosphorylated AKT at Ser473 residue and STAT3 at Tyr 705 residue in sphere-cultured SKOV3ip1 cells (Fig. 3A). Also BGJ398 decreased

the phosphorylation of AKT at Ser473 residue and STAT3 at Tyr 705 residue in a dose-dependent manner (Fig. 3B). In contrast, TAE684 and imatinib did not affect the status of phosphorylation of AKT at Ser473 residue and STAT3 at Tyr 705 residue in sphere-cultured SKOV3ip1 cells (Fig. 3C). These results demonstrated that BGJ398 suppressed the activated AKT and STAT3 signaling pathway in sphere-cultured SKOV3ip1 cells, not in monolayer-cultured SKOV3ip1 cells. Several published studies have demonstrated that JAK/STAT signaling regulates PI3K/AKT pathway in mammalian cells (35,36). Inhibition of the cell viability of sphere-cultured SKOV3ip1 cells by BGJ398 resulted in inhibition of AKT and STAT3 activation leading us to further examine whether JAK/STAT3 signaling mediates sphere culture-induced AKT and STAT3 activation in SKOV3ip1 cells. Thus, we examined whether the selective JAK tyrosine kinase inhibitor AG490 affected the activation of AKT and STAT3 status in sphere-cultured SKOV3ip1 cells. As shown in Fig. 4A, the treatment of AG490 is not able to inhibit cell growth of sphere-cultured SKOV3ip1 cells. Also, the phosphorylation of AKT and STAT3 was not inhibited by treatment of AG490 in sphere-cultured SKOV3ip1 cells (Fig. 4B). Collectively, these results indicated that BGJ398 effectively suppressed cell growth of

sphere-cultured SKOV3ip1 through the inhibition of major survival signaling molecules, AKT and STAT3 activation irrelevant to JAK/STAT3 signaling pathway.

BGJ398 synergistically inhibits the cell viability of sphere-cultured SKOV3ip1 with the combinational treatment of paclitaxel. Previous results that the metastatic microenvironment mimetic sphere culture of SKOV3ip1 enhances paclitaxel resistance led us test whether BGJ398 sensitizes paclitaxel-resistance in sphere-cultured SKOV3ip1 cells. To determine whether the combination of BGJ398 and paclitaxel can synergistically inhibit cell viability in sphere-cultured SKOV3ip1, serial diluted paclitaxel with dose-different combinations (0, 1.25 and 5 μ M) of BGJ398 were treated in sphere-cultured SKOV3ip1 and crystal violet colorimetric analysis was examined at 72 h after treatment. As shown in Fig. 5, treatment of sphere-cultured SKOV3ip1 with BGJ398 in combination with paclitaxel led to a synergistic inhibition of cell viability compared to treatment of paclitaxel alone.

Discussion

Ovarian cancer is the most lethal among gynecological cancers (1). Despite the active pharmaceutical research against ovarian cancer to date, paclitaxel is still a first-line therapeutic anticancer drug for ovarian cancer (2). Moreover, the recurrent ovarian cancer in patients due to the development of chemoresistant phenotype is the main obstacle to managing advanced ovarian cancer (37). Together with understanding of the chemoresistance molecular mechanism of ovarian cancer, developing new pharmaceutical agents will be important to overcoming of ovarian cancer. We describe here a novel antitumor activity of pan-FGFR inhibitor BGJ398 against ovarian cancer dependent on sphere culture model. Also, BGJ398 induced dissociation of the spheroid formation of ovarian cancer cells.

Although many anticancer drugs showed promising effects *in vitro* cell line model, most failed to develop into an *in vivo* model system. Application of the modified *in vitro* culture model considering *in vivo* tumor microenvironment will be important at the developmental stage of anticancer drug. It has been reported sphere culture of epithelial ovarian carcinoma cells represented the feature of *in vivo* histological differentiation rather than the cells which were cultured in conventional monolayer model (38). Moreover, the transition from monolayer culture model to sphere culture model induced changes in the expression of molecular biomarkers relevant to malignancy including cadherins, vimentin and β -catenin (38). In this study, we suggested that the activated AKT and STAT3, the key regulators in cell survival, also changed by the transition of the culture model (Fig. 1) result in sensitizing the antitumoral activity of BGJ398 against SKOV3ip1. Many studies have reported the characterization of cancer cell spheroid as a model of cancer stem cells. Ovarian cancer cell spheroids with stem cell-like phenotype have been also reported to upregulate stem cell markers, metastatic capability and acquiring chemotherapy resistance (39-41). In the present study, we did not incubate SKOV3ip1 cells with stem cell enrichment media, nor separated the side population by FACS sorting for the enrichment of ovarian cancer stem cells. AKT and STAT3

activation was demonstrated by SKOV3ip1 incubation with anchorage-independent growth. Moon and colleagues (42) have reported that PI3K/Akt and Stat3 signaling are important roles in maintenance of cancer stem cells of glioblastoma cell model. Moreover, it has been reported that STAT3 signaling pathway has a role in drug-resistance, migration, and invasion and the blockade of STAT3 potentiates chemotherapy in ovarian cancer stem cell model (43-46). Although FGFR signaling function in maintenance of cancer stem cells has been reported (47), further examinations how ovarian cancer sphere culture system upregulates cellular AKT and STAT3 activation and what the molecular mechanism of FGFR inhibitor BGJ398 in regulation of AKT and STAT3 in sphere cultured-SKOV3ip1 were needed.

BGJ398 is a selective pan-FGFR inhibitor with IC_{50} of 0.9, 1.4, 1.0 and 60 nM for FGFR1, 2, 3 and 4 in cell-free assay, respectively (48) and with potential antiangiogenic and anti-neoplastic activities (11). Cheng *et al* (49) have reported that BGJ398 inhibited the growth of E-cadherin-positive epithelial type bladder cell lines at drug concentrations of 1 μ M or lower. They also have shown that BGJ-398 did not inhibit mesenchymal type bladder cancer cells and primary tumor growth but block tumor metastasis in a mouse model *in vivo* (49). In this study, we did not observe changes of signaling molecules on EMT in SKOV3ip1 cells following sphere cultivation. Although it has been reported that SKOV3ip1 is highly malignant and has metastasis potential by overexpressing ERBB2 (50), whether FGFR may be responsible for mediating EMT signaling in ovarian cancer spheres will be an important research area. TAE684 is a selective ALK kinase inhibitor (51). TAE684 treatment induces inhibition of phosphorylation of NPM-ALK and its downstream effectors including STAT3 and STAT5 and blocked the growth of anaplastic large-cell lymphoma (ALCL)-driven and ALK-dependent cell lines (51). Imatinib is a multi-target kinase inhibitor against Abl (52), PDGF (53), and c-Kit (54) used in the treatment of various cancers, mostly BCR/ABL-mutated chronic myelogenous leukemia. It has been reported that imatinib reverses doxorubicin-resistance by repression of the NF- κ B signaling and preventing activation of STAT3/HSP27/p38/Akt survival pathway in highly active c-Abl cancer cells (55). Despite TAE684 having cytotoxic activity in monolayer-cultured SKOV3ip1 cells as well as in sphere-cultured SKOV3ip1 cells, our data showed that TAE684 and imatinib do not suppress AKT and STAT3 pathways (Fig. 3A).

The present study showing the inhibition of the SKOV3ip1 ovarian cancer growth in sphere culture system by BGJ398 treatment implies that FGFR signaling potentiated the specific antitumor target against ovarian cancer spheroids. It has been reported that RNAi-induced FGFR1/2 reduction inhibits proliferation of SKOV3 ovarian cancer cell lines *in vitro* and increases cisplatin sensitivity (56). However, we demonstrated that significant cell toxicity was not observed in BGJ398-treated monolayer-cultured SKOV3ip1 cells. Specifically, the cell viability was decreased by BGJ398 in sphere-cultured SKOV3ip1 cells (Fig. 2). Despite the absence of the expression profile of FGFR isoforms in sphere-cultured SKOV3ip1 cells, we revealed the downregulation of AKT and STAT3 activation via a pan-FGFR inhibition in sphere-cultured SKOV3ip1 cells (Fig. 3A). Furthermore, we observed cytotoxicity and dissociation of spheroids of SKOV3ip1 by treatment of

PI3K/AKT inhibitor wortmannin in SKOV3ip1 cells implying that AKT activation is critical for the cell survival in sphere-cultured SKOV3ip1 cells (data not shown). Potent therapeutic targeting of the JAK/STAT3 pathway for ovarian cancer growth inhibition has been demonstrated (57,58). Several reports have demonstrated that JAK signaling regulates PI3K/AKT pathway in cells (35,36). Recently, Wen *et al* (59) reported that JAK/STAT3 inhibition using small molecule inhibitor suppressed tumor progression and metastasis in peritoneal mouse model of ovarian cancer. Moreover, it has been reported that the inhibition of Jak2 suppresses the progression of ovarian cancer (60). In the present study, we did not investigate the defined signaling mechanism of AKT and STAT3 activation in sphere-cultured SKOV3ip1 cells. The underlying molecular mechanisms remain to be elucidated.

As shown in Fig. 2, evidence have been reported that cells formed 3D spheroids have resistance to antitumor chemotherapeutics including paclitaxel and cisplatin. This has been largely considered due to several mechanisms including a decreased penetration rate of drugs into spheroid structure and alteration of prosurvival signaling pathway (61,62). Cumulative evidence have showed that β -integrin signaling is important in the maintenance of ovarian cancer spheroid. Casey *et al* (63) reported that ovarian cancer spheroid formation and the adhesion of spheroids to extracellular matrix components is a β -integrin-dependent event. Sodek *et al* (64) demonstrated that the overexpression of β 1-integrin induces compact spheroid formation and invasive behavior in ovarian cancer cells. Yoshida *et al* (62) reported that laminin-1-derived synthetic peptide AG73 enhances the expression of β 1-integrin and induced the activation of downstream effector genes including MAPK, ERK and AKT. Therefore, we cannot rule out the possibility that the interference of the β -integrin pathway by BGJ398 may exist and contribute to BGJ398-induced cytotoxicity in sphere-cultured SKOV3ip1 cells.

Solid tumors on primary tissue were organized upon its specific tumor microenvironment with cell-to-cell or cell-to-extracellular matrix association. Unlike other cancers, ovarian cancer metastasis is distinctly developed by dissemination into peritoneal cavity and associated with the ascitic fluid to form metastatic microenvironment (65). Within ascitic fluid, ovarian cancer cells exist as individual cells or multicellular aggregates with absence of anchorage-dependent signaling. In ovarian cancer-specific microenvironment, metastases aggregates have an enhanced resistance to anticancer drugs, including paclitaxel (66). In the present study, BGJ398 sensitized the cell cytotoxic activity with a combination treatment of paclitaxel in sphere-cultured SKOV3ip1 cells implying a possibility of new therapeutic approaches for targeting against ovarian cancers spheroid regarding ovarian tumor microenvironment (Fig. 5).

In conclusion, the present study suggests that pan-FGFR inhibitor BGJ398 is a potent chemotherapeutic agent for ovarian cancer spheroid. Our data also indicated that FGFR may have a unique prosurvival role in the spheroid maintenance of ovarian cancer. This study gives us a more comprehensive insight into 3D sphere cell culture model during drug development considering ovarian cancer specific microenvironment and the antitumor activity of FGFR inhibitor against metastatic ovarian carcinoma.

Acknowledgements

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2015R1D1A1A01060688).

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